REMARKS

The claims

Previous claims 2, 3, 5, 10-12 and 17-19, and newly added claims 20-26 are currently under consideration. Claims 5 and 17-19 are amended to address points raised in the outstanding Office Action. The amendments and the new claims are fully supported in the specification. Claims 6-8 and 13-16 have been withdrawn from consideration.

Claims 17, 2, 3, 10, 11, 19 and 25 are directed to a purified nucleic acid that comprises the sequence of SEQ ID NO: 1, or a complete complement thereof, and to detection methods using the nucleic acid.

Claims 18 and 5 are directed to a purified nucleic acid that consists of a fragment of the sequence of SEQ ID NO: 1 (wherein said fragment hybridizes specifically with a nucleic acid molecule having a sequence that is completely complementary to SEQ ID NO: 1), or a complete complement thereof .

Claims 21, 20, 22-24 and 26 are directed to a purified nucleic acid that comprises a sequence that hybridizes specifically with a nucleic acid molecule consisting of the sequence of SEQ ID NO: 1, or a complete complement thereof, and to detection methods using the nucleic acid.

1449 Form

Applicants request that another copy of initialed Form 1449 be sent with the next communication from the Examiner.

The rejection under 35 USC 112, second paragraph:

Contrary to the assertion in the Office Action, the term "abnormally high" is, indeed, discussed in the specification. See, *e.g.*, page 10, lines 20-24:

> "An 'abnormally high' level, or content, of the nucleic acid transcript, as used herein, relates to a ratio of the level of PB39 transcript to the level of beta-actin transcript that is preferably about two times or more higher in the sample of the cancerous epithelium than that found in a set of samples taken from normal epithelium, as expressed by a mean value found therein."

The specification clearly discloses examples of abnormally high expression (overexpression) of PB39 transcripts in neoplastic prostate tissue (prostatic intraepithelial neoplasia) compared to normal tissue from a prostate gland. See, *e.g.*, page 9, line 30 through page 10, line 2 and Example 3.

The rejections under 35 USC 112, paragraph one

With regard to the rejections under 35 USC 112, paragraph one (written description and scope of enablement), based on the recitation of the term "a nucleic acid molecule that comprises a fragment of the sequence of SEQ ID NO:1" (which hybridizes specifically with a nucleic acid having the sequence of SEQ ID NO:1), applicants maintain that this recitation is definite, and defines a scope of fragments that is fully enabled by the specification. Note that amended claim 18 recites, *i.a.*, a nucleic acid that consists of a fragment of the sequence of SEQ ID NO:1 ...

Written description: One of skill in the art, upon learning of the sequence of SEQ ID NO:1, would immediately recognize a variety of fragments that consist of a fragment of the sequence of SEQ ID NO:1, and thus would be in possession of the genus of fragments. Moreover, and supplementally, at least three fragments of SEQ ID NO:1 (primers) are explicitly disclosed in the specification (see, *e.g.*, SEQ ID NOs 7, 8 and 10 on page 4, lines 9-11). These species provide a representative number of, and therefore define, the genus of claimed fragments. Thus, the specification contains written description of the genus of claimed fragments. In any case, claim 5, which recites nucleic acids consisting of SEQ ID NOs 7, 8 or 10, is clearly described by the specification.

Enablement: One of skill in the art would know how to make and use any of the species of fragments for which written description is found. Clearly, any nucleic acid fragment of sufficient length which consists of a sequence of SEQ ID NO:1, or a complete complement thereof, would hybridize specifically to a nucleic acid having a complete complement of SEQ ID NO:1, or SEQ ID NO:1, respectively. Only one use is required to satisfy the utility requirement for a compound, such as a nucleic acid fragment of the invention. In the present case, the ability of the fragment to hybridize specifically to a nucleic acid having SEQ ID NO:1, or to a complete complement thereof, and thereby to detect prostate cancer in a subject, is sufficient to satisfy the utility

requirement. Such a functional requirement is recited in, *e.g.*, claim 18. The discussion in the Office Action of the effects of mutations on protein function bears no relation to the presently claimed nucleic acid fragments.

With regard to the rejections based on the terms "complementary to", under 35 USC 112, paragraph one paragraph one (written description and scope of enablement), applicants maintain that it would be clear to a skilled worker that a sequence which is "complementary to" another sequence is "*completely* complementary to" that sequence. See, *e.g.*, the attached definition from *A Dictionary of Genetics* (Appendix A), which defines a "complementary base sequence" as "a sequence of polynucleotides related by the base-pairing rules. For example, in DNA a sequence A-G-T in one strand is complementary to T-C-A in the other strand. A given sequence defines the complementary sequence." Nevertheless, in a effort to expedite prosecution, the claims have been amended to recite that the sequences are "*completely* complementary." This amendment does not narrow the scope of the claims.

With regard to the rejections under 35 USC 112, paragraph one (written description and scope of enablement), based on the recitation of the term "hybridizes specifically," applicants maintain that it would be clear to a skilled worker from reading the specification, and from the art-accepted meaning of the term, what is meant by the term "hybridizes specifically," and how to use fragments that exhibit such high specificity of hybridization to detect prostate cancer.

The specification is replete with examples of specific hybridization. For example, page 16, lines 14 to page 17, line 10, figure 1 and Table 1 illustrate analyses in which PB39-specific primers were used to specifically PCR amplify sequences found in prostate tumors but not in control tissues. Clearly, the hybridization conditions used for the PCR primer annealing steps resulted in specific hybridization of the primers to the template. Furthermore, Examples 4 and 5 (page 17) and Figures 2 and 3 illustrate specific annealing of probes in Northern blot analysis. Clearly, specific hybridization conditions were used to identify specific bands on the gels. One of skill in the art would recognize that a reasonable interpretation of the term "specific hybridization" is hybridization in

which a nucleic acid binds preferentially to a target of interest. The allegation by the Examiner at page 5, third full paragraph of the Office Action that "specific hybridization" would be interpreted by a skilled worker to include even non-specific binding is untenable. Furthermore, the allegation at page 5, first full paragraph of the Office Action that the claimed fragments could be "only a few nucleotides" is unwarranted in view of the functional limitation that the fragment hybridize specifically to a nucleic acid consisting of SEQ ID NO: 1.

The Examiner, on page15, lines 3-9 of the Office Action, cites a passage from Sambrook *et al.*, 1989 which teaches that a skilled worker can easily determine hybridization conditions that "suppress hybridization of [a] probe to incorrect sequences." The discussion in Sambrook supports applicants' assertion that skilled workers at the time the invention was made were aware of what is meant by the term specific hybridization, and knew how to determine conditions (without undue experimentation) to achieve such specific hybridization.

With regard to the rejections under 35 USC 112, first paragraph (scope of enablement), based on the recitation of "tissue or fluid" from a subject:

Contrary to the allegation in the Office Action, a skilled worker reading the present application would recognize that claim 19 (and claim 20, and claims dependent on claims 19 or 20), do not read on a method for detecting prostate cancer by detecting the levels of SEQ ID NO: 1 in "any tissue" or "any fluid" or "any bodily fluid." Rather, a skilled worker would recognize those tissues or body fluids which would be suitable for such analysis. The specification provides guidance as to which tissues or fluids would be suitable. See, *e.g.*, page 10, lines 25-30:

> "In performing this method, the sample from a subject may be a biopsy sample drawn from the prostate gland of the subject. ... Alternative samples may be a body fluid from the subject, including but not limited to blood, urine and seminal fluid. Generally, sampling methods and choices are well known to workers of skill in the art such as urologists and oncologists."

Further support that skilled workers at the time the invention was made were aware of suitable body fluid samples is provided by the references in Appendix B. Itoh *et*

al. (1998) *Renal Failure* 20, 235-241 disclose sampling urine for the presence of a prostate tumor marker, BFP (see, *e.g.*, the Abstract and the Introduction); and Lumbardo *et al.* (1997) *Steroids* 62, 682-685 disclose sampling urine for the presence of the prostate cancer marker, 5α-reductase type 2 (see, *e.g.*, the Title and Abstract). In addition, the post-filing review article by Tricoli *et al.* (2004) *Clinical Cancer Research* 10, 3943-3953 refers to pre-filing papers which disclose methods for testing, *e.g.*, human serum, urine, seminal fluid and histological specimens for prostate cancer markers.

With regard to the rejections under 35 USC 112, first paragraph (scope of enablement), based on the recitation of "detecting prostate cancer" in a subject:

The Examiner appears to allege that the specification is only enabled for methods for detecting *primary* prostate tumors. However, the Examiner has not met her burden of proving the allegation that expression of the claimed sequence is lost during the progression during metastasis, and that that the claimed method is thus allegedly unable to detect *metastacized* prostate tumors.

The Examiner cites references in which prostate tumor markers are allegedly down-regulated during metastasis. However, for at least two reasons, the cited references do not cast doubt that PB39 would continue to be expressed or over-expressed during metastasis:

(1) As shown in the present application, PB39 is up-regulated in primary prostate tumors compared to normal cells. The cited references do not disclose markers that are up-regulated in primary tumors, then down-regulated during metastasis. Thus, the cited references do not bear on the present situation, in which a gene (PB39) is up-regulated in tumors compared to normal cells.

(2) The observations in the present study are performed on primary tissues - purified epithelial cells which are micro-dissected from human tissues. By contrast, the cited references report on studies performed with cell lines, clones or transfection models. These studies may not reflect events which occur *in* vivo.

More specifically:

Zhau 94: Most of the data in this paper appear to be from cell lines, clones or transfection models; genetic changes are reported to occur during passages of cells in

culture. The Examiner has not pointed to any examples from the reference in which such changes are observed in primary tumors (compared normal primary cells). Moreover, the Examiner has not pointed to any example in the reference of a marker that is over-expressed in tumors, and then down-regulated during metastasis.

Gingrich 96 reports a loss of normal E-cadherin expression comparing tumor vs metastasis, in a transgenic mouse model. However, E-cadherin over-expression is *not* associated with prostate tumors in humans. In fact, it is the loss (down-regulation) of E-cadherin which is associated with the progression of human prostate cancer. See, *e.g.*, references 7 and 8 cited in the Gingrich paper.

Ren 98 reports that lysyl oxidase mRNA is down-regulated in metastasis vs primary tumors. However a) the gene is not shown to be over-expressed in *primary* tumor vs normal human prostate cells; b) the data refer in part to primary vs metastatic *cell lines* that are stimulated *in vitro* with growth factors or c) refer to *in situ* hybridization of mouse model samples. Furthermore, and perhaps more importantly, the Examiner has not pointed to any instances from the reference in which a gene that is up-regulated in tumor vs normal cells is then down-regulated during metastasis.

Kibel 00 reports deletions that play a role in inactivating a gene in metastasis vs primary tumor. The gene however is not shown to be over-expressed in tumors vs normal cells. On the contrary, it follows the traditional inactivation model of a tumor suppressor gene in the progression of tumors.

Cheung 02 shows cDNA arrays for the analysis of thousands of genes in tumor vs metastatic liver tissues, with the aim of determining gene expression patterns characterizing primary vs metastatic tumors. Several genes are reported to be down-regulated in metastasis vs primary tumors. However, the Examiner has not pointed to any instances of down-regulated genes which had been up-regulated in tumors compared to normal cells.

See also claims 25 and 26, which recite a method wherein the prostate cancer is a primary tumor.

In view of at least the preceding amendments and arguments, it is requested that the rejections under 35 USC 112 be withdrawn.

The rejections under 35 USC 102

1. The rejection under 35 USC 102(b) over the random sequences in the Boehringer Mannheim Biochemicals catalog:

The Office Action alleges that the random 6-mer sequences disclosed in the Boehringer Mannheim Biochemicals catalog anticipate claims directed to a purified nucleic acid molecule that is "complementary" to SEQ ID NO: 1 (claims 17). The reference does not anticipate claims directed to a nucleic acid molecule that is "*completely* complementary" to SEQ ID NO: 1. See amended claim 17.

The Office Action also alleges that the random 6-mer sequences disclosed in the Boehringer Mannheim Biochemicals catalog anticipate claims directed to "a purified nucleic acid molecule that comprises a fragment of SEQ ID NO: 1, wherein said fragment hybridizes specifically with SEQ ID NO: 1 (claim 18)." It is noted that amended claim 18 recites, *i.a.*, a nucleic acid molecule that consists of a fragment of the sequence of SEQ ID NO: 1, wherein said fragment hybridizes specifically with a nucleic acid molecule having a sequence that is completely complementary to SEQ ID NO: 1, or fragments which are completely complementary to said fragments.

The random 6-mer sequences of the Boehringer Mannheim Biochemicals catalog are clearly too small to hybridize specifically to a nucleic acid that is completely complementary to SEQ ID NO:1, or to a sequence comprising the sequence SEQ ID NO: 1. In fact, the specification teaches (*e.g.*, at page 14, lines 13-15) a procedure in which such random hexamers were used to generate a *random* collection of cDNAs by RT-PCR. (The cDNAs were then further characterized, using more specific primers.) The 6-mers were used for their ability to bind randomly to a target, rather than specifically. Therefore, the instant claims which recite fragments that exhibit the functional limitation that they hybridize specifically to a particular nucleic acid (*e.g.*, amended claim 18) are distinguished from, and not anticipated by, the 6-mers of the cited catalogue reference. As stated on page 10, lines 10-13 of the specification, fragments used in hybridizations, *e.g.* in Northern analysis, must be "long enough to hybridize effectively with the target sequence in the sample being probed." One of skill in the art would recognize that such probes must be more than 6 bases in length.

2. The rejection under 35 USC 102(a) over WO98/21328-A2:

The Office Action alleges that a sequence disclosed in WO98/21328-A2 anticipates claims directed to a purified nucleic acid that is "complementary" to SEQ ID NO: 1 (claims 2, 3 and 17).

The reference is alleged to disclose a sequence which is 99.5% similar to SEQ ID NO: 1. Thus, the cited sequence is not *completely* complementary to the sequence of SEQ ID NO: 1, as recited, *e.g.*, in amended claims 2, 3 and 17. Thus, the reference does not disclose every material element of the nucleic acid of claims 2, 3 and 17, and so does not anticipate those claims (*In re Marshall*, 198 USPQ 344 (CCPA 1978)).

The Office Action also alleges that claims such as claims 18 and new claim 22, which recite a sequence that "hybridizes specifically" to a nucleic acid comprising SEQ ID NO: 1, are anticipated by the reference. Amended claim 18 recites, *i.a.*, a nucleic acid molecule that consists of a fragment of the sequence of SEQ ID NO: 1, wherein said fragment hybridizes specifically with a nucleic acid molecule having a sequence that is completely complementary to SEQ ID NO: 1. Clearly, the PCT reference does not anticipate the claimed fragments. As for new claim 21, which recites: "(A) a nucleic acid molecule that comprises a sequence that hybridizes specifically with a nucleic acid molecule consisting of the sequence of SEQ ID NO: 1, or (B) a nucleic acid that comprises a sequence that is completely complementary to the sequence of said nucleic acid molecule (A)," the attached Declaration under 37 CFR 1.131 establishes a date of reduction to practice for at least this aspect of the claimed invention and/or for the relevant content of WO98/21328-A2, prior to the May 22, 1998 earliest effective date for the WO98/21328-A2 reference. *In re Spiller*, 182 USPQ 614 (CCPA 1974).

3. The Office Action alleges that sequences disclosed in Hudson, Genbank Accession Number G22380, anticipate claims directed to a purified nucleic acid that is "complementary" to SEQ ID NO: 1 (claims 2, 3 and 17). Hudson does not teach a nucleic acid which is *completely* complementary to SEQ ID NO: 1 (*e.g.*, amended claims 2, 3 and 17).

The Office Action also alleges that sequences disclosed in Hudson anticipate claims directed to a "purified nucleic acid molecule that 'comprises' a fragment of SEQ

ID NO: 1, wherein the fragment 'hybridizes specifically' with SEQ ID NO: 1, or a 'complement' thereof" (*e.g.*, claims 5 and 18).

Hudson discloses a sequence of 321 bases. Although Hudson's sequence may contain sequences that are found in SEQ ID NO: 7 and SEQ ID NO: 10, Hudson does not disclose a sequence that *consists of* the SEQ ID NO: 7 or 10. Absent such a disclosure, the reference does not anticipate the present claims (*e.g.*, amended claims 5 and 8).

In view of at least the preceding amendments and arguments, it is requested that the rejections under 35 USC 102 be withdrawn.

In view of the preceding arguments and amendments, it is believed that the application is in condition for allowance, which action is respectfully requested.

Should any additional fee be deemed due, please charge such fee to our Deposit Account No.22-026, referencing docket number 31978-202420 and advise us accordingly. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Date: **November 5, 2004**

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APPENDIX A

A DICTIONARY OF GENETICS

Fifth Edition

ROBERT C. KING
Northwestern University

WILLIAM D. STANSFIELD
California Polytechnic State University

New York *Oxford*
Oxford University Press
1997

Oxford University Press

Oxford New York
Athens Auckland Bangkok Bogota Bombay
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5 7 9 8 6 4

Printed in the United States of America
on acid-free paper

reduce competition. Also known as *Gause's principle.*

complement a group of at least nine proteins (C1, C2, . . . C9) normally found in vertebrate blood serum that can be activated immunologically (by antibodies of immunoglobulin classes IgG or IgM) or nonimmunologically (by bacterial lipopolysaccharides and other substances) through an alternate (properdin) pathway. Activation of the system involves sequential conversion of proenzymes to enzymes in a manner analogous to the formation of fibrin through the blood-clotting sequence. Some activated complement components enhance phagocytosis (opsonic activity), some make antigen-antibody-complement complexes "sticky" and cause them to become affixed to endothelial tissues or blood cells (serological adhesion or immune adherence), some cause release of vasoactive amines from blood basophils or tissue mast cells (anaphylotoxins), and some cause dissolution of bacterial cells (bacteriolysis). *See* **HLA complex.**

complementarity-determining region the segment of the variable region of an immunoglobulin or T cell receptor molecule that contains the amino acid residues that determine the specificity of binding to the antigen. *See* **paratope.**

complementary base sequence a sequence of polynucleotides related by the base-pairing rules. For example, in DNA a sequence A-G-T in one strand is complementary to T-C-A in the other strand. A given sequence defines the complementary sequence. *See* **deoxyribonucleic acid.**

complementary DNA *See* **cDNA.**

complementary factors complementary genes.

complementary genes nonallelic genes that complement one another. In the case of dominant complementarity, the dominant alleles of two or more genes are required for the expression of some trait. In the case of recessive complementarity, the dominant allele of either gene suppresses the expression of some trait (i.e., only the homozygous double recessive shows the trait).

complementary interaction the production by two interacting genes of effects distinct from those produced by either one separately.

complementary RNA *See* **cRNA.**

complementation appearance of wild-type phenotype in an organism or cell containing two different mutations combined in a hybrid diploid or a heterokaryon. *See* **complementation test.**

complementation group mutants lying within the same cistron; more properly called a *noncomplementation group.*

complementation map a diagrammatic representation of the complementation pattern of a series of mutants occupying a short chromosomal segment. Mutually complementing mutants are drawn as nonoverlapping lines, and noncomplementing mutants are represented by overlapping, continuous lines. Complementation maps are generally linear, and the positions of mutants on the complementation and genetic maps usually agree. A complementation map is thought to show sites where lesions have been introduced into the polypeptides coded for by the DNA segment under study.

complementation test the introduction of two mutant chromosomes into the same cell to see if the mutations in question occurred in the same gene. If the mutations are nonallelic, the genotype of the hybrid may be symbolized (*a+/+b*). The wild phenotype will be expressed, since each chromosome "makes up for" or "complements" the defect in the other. *See also* **allelic complementation, *cis-trans* test.**

complete dominance *See* **dominance.**

complete linkage a condition in which two genes on the same chromosome fail to be recombined and therefore are always transmitted together in the same gamete.

complete medium in microbiology a minimal medium supplemented with nutrients (such as yeast extract, casein hydrolysate, etc.) upon which nutritional mutants can grow and reproduce.

complete metamorphosis *See* **Holometabola.**

complete penetrance the situation in which a dominant gene always produces a phenotypic effect or a recessive gene in the homozygous state always produces a detectable effect.

complete sex linkage *See* **sex linkage.**

complexity in molecular biology, the total length of different sequences of DNA present in a given preparation as determined from reassociation kinetics; usually expressed in base pairs, but the value may also be given in daltons or any other mass unit.

complex locus a closely linked cluster of functionally related genes: e.g., the human hemoglobin gene complex or the *bithorax* locus in *Drosophila.* *See* **pseudoalleles.**

composite a plant of the immense family Compositae, regarded as comprising the most highly developed flowering plants. It includes species such as the daisy and sunflower.

composite transposon a DNA segment flanked on each end by insertion sequences (*q.v.*), either or both of which allow the entire element to transpose.

Kribben et al.

lcitriol in a proximal tubular cell

induced nephrotoxicity and mod-
33, 1992.

Renal Failure, 20(2), 235–241 (1998)

Basic Fetoprotein in Normal and Pathologic Urine

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ABSTRACT

We developed a latex agglutination nephelometric immunoassay for urinary basic fetoprotein (BFP) that functioned well and had good specificity, precision, and recovery. Reference intervals started below 0.5 μg/L, the lower limit of the range of sensitivity of the assay, and went up to 7.0 μg/L at the 97.5th percentile without age- or sex-related variation, in accordance with the NCCLS guidelines. BFP was unstable at pH 5.0 at 4°C and 25°C. The western blot method showed BFP found in the semen to be structurally identical to purified BFP from hepatoma ascites, in which concentration ranged from 94.2 to 145.2 μg/L and, further, to have the same molecular weight and reactivity with a monoclonal antibody. BFP levels were elevated in cases urinary BFP concentration included ureter stone, infection, and prostate and bladder cancer. Moreover, BFP concentration correlated closely with that of α_2-macroglobulin, indicating that BFP is probably secreted locally in close pathophysiologic association with post-renal hemorrhage. We thus conclude that BFP is a urinary nonspecific marker for inflammation or tumor. The best indication for BFP as a tumor marker may be follow-up when diagnosis of genitourinary cancer is definite.

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INTRODUCTION

Basic fetoprotein (BFP)is a low molecular weight nonglycoprotein with a molecular mass of 55 kDa and an isoelectric point (pI) ranging from 8.5 to 9.15 that was first isolated from ascites of hepatoma by Ishii (1). Isoelectric focusing has shown the pI to be relatively low when malignancies are present. After preparation of monoclonal antibodies against BFP of low pI, close to 8.5, an enzyme-linked immunosorbent assay for serum BFP was established in an attempt to use serum BFP levels to detect the presence of cancer. Serum BFP is a well-established nonspecific marker for cancer in Japan, where it is used mainly to detect prostate cancer and small cell cancer of the gall bladder, kidney, and lung (1,2).

The significance of this protein as a urinary tumor marker is a subject of keen interest today. In some clinical studies, levels of urinary BFP were frequently elevated in patients with bladder, renal pelvic, urethral, or prostate cancer (3,4).

One major problem remained, however: No complete fundamental laboratory investigation of urinary BFP had been conducted, even though its potential clinical significance had been reported. We thus recently developed a reliable automated latex agglutination particle assay for urinary BFP. Here we evaluate the function of this assay, and investigate preanalytical conditions specific to urine, thus re-evaluating the clinical application of this protein under physiologic and pathologic conditions.

MATERIALS AND METHODS

Samples. Urine was obtained from 152 normal, healthy adults ranging in age from their third to their seventh decades at the Jichi Medical School Health Care Center, and pathologic urine was collected at random at the clinical laboratory of this institution from 300 outpatients of the department of nephrology and urology. Also ten more semen samples were obtained with informed consent from healthy males in their third decade. In order to investigate BFP of genital tissue origin, three-divided voided urine was obtained from 10 men and 6 women, following a previously reported protocol (5).

Determination of urinary BFP and other parameters. An automated latex agglutination nephelometric immunoassay (LX-3000, Eiken Co. Ltd., Tokyo) was used to measure urinary protein levels. The basic principle behind the assay and the procedures used have been reported (6) elsewhere. Purified BFP was used as a standard substance after determining its optical density, 280 nm, assuming $E^{1\%\ 1cm} = 12\ 0.936$. Four different clones of monoclonal antibodies, designated K1, 5C2, 5C4, and 5C6, were used to coat polystyrene latex particles to capture BFP. Urine samples, usually undiluted, but diluted 1:10 with dilution buffer (50 mM Hepes buffer, pH 7.4, containing 0.9% NaCl, 0.1% NaN^3, and 0.1% BSA) when BFP level was over 50 μg/L, were reacted with a suspension containing antibody-coated latex particles in the reaction buffer (0.1 M Hepes buffer, pH 7.4, containing 0.9% NaCl, 0.1% NaN_3, 0.5% BSA) for 560 seconds. Degree of latex agglutination was measured 170 and 560 seconds later at the wavelength of 660 nm. For elucidating pathogenesis, and for local diagnosis in prerenal, renal, and post-renal pathophysiologic dysfunction, the urine protein profile was determined by measuring the five key proteins simultaneously. These comprised albumin and IgG, as glomerular markers; α_1-microglobulin, as a tubular marker; α_2-

macroglobulin, as a post-renal hemorrhage or highly advanced glomerular dysfunction marker; and interleukin 8, as an inflammatory marker (7,8).

Western blotting. Sodium dodecyl sulfate gel electrophoresis was performed at 25 mA for 3 hours on 12.5% polyacrylamide gel. Electric transfer of separated proteins to a nitrocellulose membrane was conducted at 100 mA and 12 V for 1.5 hours. The indirect immunoperoxidase method was used to detect BFP on the membrane (5).

RESULTS

Function of the assay. Either of two standard curves, set for high and low concentrations of BFP, can be selected automatically by using the value obtained from, the given sample. Actual analytical range was 1.5 to 50 μg/L, and sensitivity was 0.5 μg/mL. In dilution tests, levels in urine diluted to three different concentrations fell on curves that were parallel to the standard curve. Three urine specimens were measured ten consecutive times on the same day.

Intra-assay coefficients of variation (CV) ranged from 1.4% to 2.8%. Further, three different samples were measured on each of 7 consecutive days. Inter-assay CVs were 2.9% to 6.5%. In the recovery test, purified BFP was added to three samples with different concentrations. Recovery rate ranged from 99.1% to 102.8%, and no nonspecific adhesion on glass or plastic containers was observed. Addition of various concentrations of bilirubin, hemoglobin, and lipid to the urine samples did not interfere with the assay.

Stability of BFP in normal urine. Purified BFP was added to two normal urines that differed in pH and temperature, and the concentration of BFP was followed for 10 days. BFP lost its antigenicity at pH 5.0 within one day at both 4°C and 25°C, but antigenicity was stable for up to 10 days when urine samples were held at intermediate temperatures (Fig 1). There was no further degradation of BFP at pH 5.0 at any temperature, however, when urine was mixed with sample buffer at the time of collection (data not shown).

Reference range of BFP. Reference range of BFP was determined in accordance with NCCLS guidelines (9). Because the distribution of BFP is nonparametric, and because BFP levels in 10% of the samples were too low to be detected by the assay, concentration at the upper 97.5th percentile, 7 μg/L, was defined as the upper limit of the reference intervals, while the value at the 2.5th percentile was below the sensitivity of the assay. There was no age- or sex-related variation.

Discovery of BFP in the semen. Concentration of urinary BFP in three-divided urines collected from 12 healthy males in their third decade was highest in the first, lower in the second, and lowest in the last voided fractions, in eight of the samples examined, indicating that BFP is present in the urethra and is washed out by voiding. Concentration of BFP in the semen was markedly higher than urinary concentration in 10 semen samples that were thus investigated: average concentration was 117.8 μg/L; the range was 94.2 to 145.2 μg/L. On western blots, a single clear band at the same migrate site as that produced by purified BFP reacted with anti-BFP mouse monoclonal antibody (Fig 2). Effects of semen on urinary concentration of BFP was thus investigated by measuring BFP in initially voided urine of the same males for 3 days before and after ejaculation. BFP concentration varied widely between individuals and with time of collection. BFP level was higher in urine collected after ejaculation than in that collected before ejaculation in four cases, but all levels fell within the reference range of proteins.

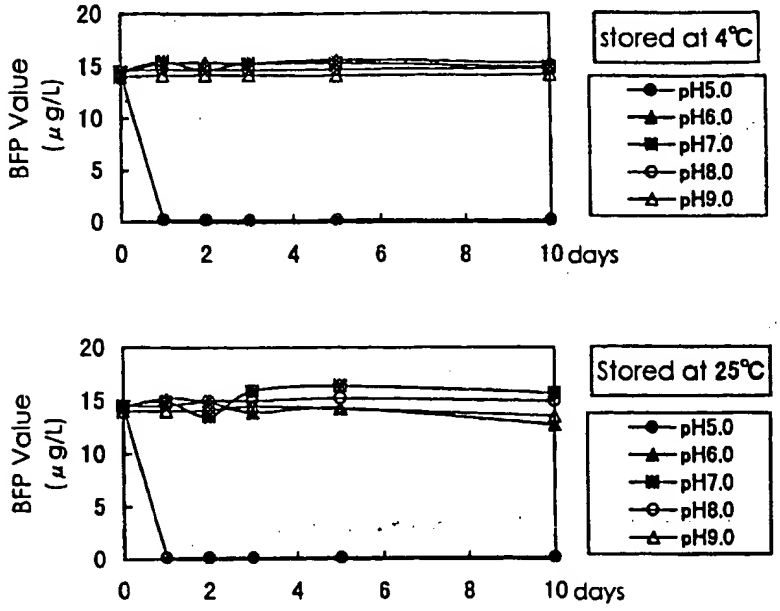

Figure 1. Instability of urinary BFP in normal urine of pH 5.0 held at 4°C and 25°C.

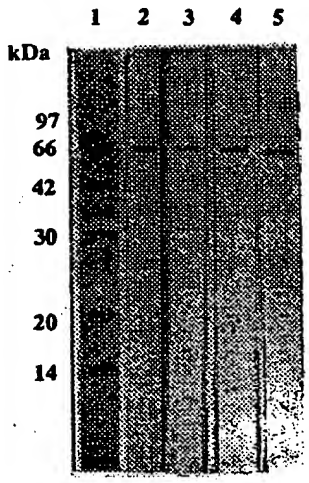

Figure 2. Detection of BFP in semen by western blotting. Lane 1: Marker proteins, Lane 2: Purifed BFP, Lane 3–5: Seminal plasma

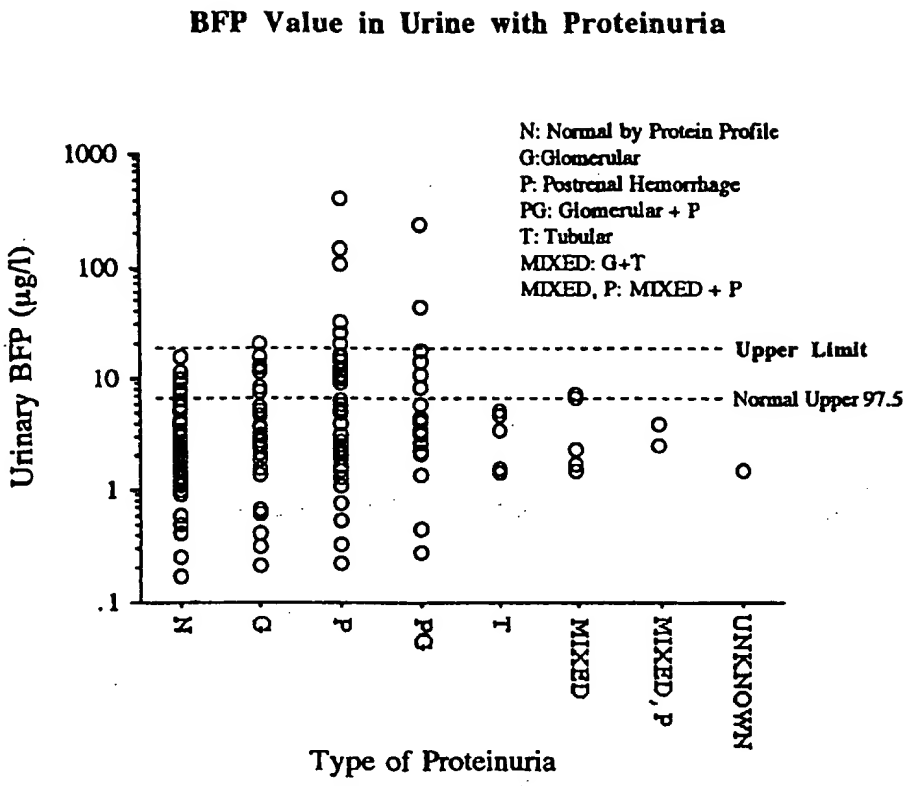

Figure 3. Urinary BFP values in pathologic conditions, chemically classified by urine protein profiles.

BFP in pathologic conditions. Elevated levels of urinary BFP in urine samples collected randomly in the clinical laboratory were associated with an array of diseases, and the correlation was especially close in cases of urogenital diseases: infections, ureter stones, bladder carcinomas, and prostate cancers, among others. of the markers investigated, only α2-macroglobulin correlated closely with BFP (r = O.71; $p < 0.01$). Number of white blood cells, red blood cells, and bacteria in urinary sediments, as well as levels of proteins, showed no correlation. A urine profile system was used to determine underlying pathogenesis and affected site. In disorders classified by protein profiles, only one case, post-renal hemorrhage confirmed by the patient's clinical record, was above the upper limit of the normal range. There was no elevation of BFP level in single glomerular or tubular disorders (Fig 3).

DISCUSSION

The latex agglutination particle assay reported here functioned well. It detected BFP specifically and sensitively and had good precession and recovery in both physiologic and pathologic urine.

As is the case with most of the proteins present in acid urine, BFP is readily broken down at pH 5.0, even when the urine is held at 4°C. About 25% of samples examined in our clinical laboratory were between pH 5 and pH 6. Loss of immunoreactivity at time of collection should, under ordinary sampling conditions, lead to underestimation of clinical results. Although degradation is inevitable at time of sampling, further degradation can be prevented by adjusting pH and diluting the sample with Hepes buffer containing BSA. Basic proteins like BFP and cystatin C are structurally stable in alkaline semen but may become unstable when pH is low. BFP was probably digested by urinary acid proteases. Freeing from protease effects, adjusting pH to neutral, and, perhaps, the 'stabilizing effect' of BSA on BFP may block further degradation. In this study, neither aggregation nor nonspecific binding on the container was a cause.

BFP structurally identical to purified BFP was first discovered in the semen, and because its concentration in the semen was 10 to 20 times higher than in the serum, the effect of semen BFP on urinary BFP concentration was investigated. Although an elevation was observed in 4 of the 10 cases examined, all were within the reference range, although they varied widely. There were no age- or sex-related differences in urinary level. Thus the authors believe that ejaculation has little or no effect on urinary BFP levels. Nevertheless, in males of reproductive age, a careful history should always be taken in order to rule out the participation of BFP or genital tissue origin.

Previous studies have found no correlation between serum and urinary BFP-S concentratioins (2, 3). Even though its molecular weight is low, behavior of this protein differed from that of ordinary low molecular weight proteins, independent of glomerular filtration rate. These findings were partly supported by the present study, in which there was no correlation between the concentration of urinary BFP and the concentration of albumin, IgG, or other urinary glomerular markers. Urinary BFP concentration thus reflects only physiologic and pathologic events that are localized in the genitourinary tissues and is independent of serum BFP level.

Clinically, the urinary concentration of BFP was highly elevated in urogenital disorders, and, in the cases studied here, the concentration of urinary BFP and α_2-macroglobulin were closely associated. Analysis by profiling urinary proteins showed that only post-renal hemorrhage was clearly related to an increase in BFP (Fig 4); renal glomerular or tubular dysfunction were not. This suggests that BFP may be associated with tumors and other hemorrhagic events in localized urogenital tissues. Recent immunohistologic studies suggest that BFP is present in transitional cells in the bladder and ureter (unpublished data). This clearly supports the view that urinary BFP can be elevated as a result of increased synthesis and secretion by tumor cells of transitional-cell origin (1-3). Moreover, cell damage and repair associated with hemorrhage, also in association with ureter stones or infection, can also either release BFP locally or increase its synthesis, thus providing another source of elevated urinary BFP.

We thus conclude that BFP is a urinary nonspecific marker of inflammation or tumor. Thus the measurement of urinary BFP alone cannot be used to screen for cancer. However, the measurement of urinary BFP may be of value in the follow up of patients with known genitourinary cancers.

ACKNOWLEDGMENT

This work was supported by Grants-in-Aid from the Ministry of Education and Culture (Project Nos. 076724% and 08672648) and the Kurozumi Medical Foundation.

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ELSEVIER

Preliminary evaluation of 5α-reductase type 2 in urine as a potential marker for prostate disease

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The objective of this study was to find a biomarker, easily detectable and measurable, that could be useful to the physician for the diagnosis and management of prostate cancer. An immunoaffinity-purified polyclonal antibody to the 5α-reductase type 2 isozyme was prepared following standard procedures in New Zealand White rabbits. One hundred and seven urine samples were examined for the presence of this isozyme by Western blot, dot blot, and enzyme-linked immunosorbent assay assays. In a control group of 91 subjects (46 females and 45 males) with no history of prostate disease, only 1 female tested positive. In a test group of 16 males, 4 males with adenocarcinoma of the prostate under treatment with lupron/flutamide tested negative. Four males with untreated adenocarcinoma of the prostate tested positive. Two males with transitional cell carcinoma invading the prostatic ducts and two males with basal cell hyperplasia of the prostate with intraductal dysplasia tested positive. These results support the need for an extended study to explore the use of the Western blot or the simple dot blot and enzyme-linked immunosorbent assays for the detection of 5α-reductase type 2 in urine as a potential marker for prostate disease. (*Steroids* **62**:682–685, 1997) © *1997 by Elsevier Science Inc.*

Keywords: prostate; 5α-reductase; biomarker; urine

Introduction

The prevalence of prostate cancer and its increasing incidence with increasing life expectancy in North American men has been well documented.[1] The need for the development of new prostatic tumor markers that could be useful to the physician for the management of prostate cancer has been discussed previously.[2, 3] A recent study by Moreno et al.[4] identifying prostatic cells in the blood of patients with metastatic prostate cancer prompted a search in our laboratories for the 5α-reductase type 2 isozyme in the peripheral circulation of patients with this disease. Polyclonal antibodies were prepared to a peptide representing amino acids 28–43 of the 5α-reductase type 2 isozyme. Using immunoaffinity-purified antibodies, these studies led to the immunochemical detection of 5α-reductase type 2 in human serum and suggested that this isozyme was linked to an immunoglobulin.[3] In this study, we report the immunochemical detection of 5α-reductase type 2 in the urine of patients with prostatic disease. Although enzyme activity was not used as a criteria in these studies to characterize the 5α-reductase type 2 isozyme, the use of a peptide segment of a protein or enzyme to generate antibodies to that protein or enzyme and the subsequent use of the immunoaffinity-purified antibodies to recognize that protein or enzyme in biological media with a high degree of probability is a well accepted procedure in the scientific community.[5]

Experimental

Antibody preparation

The preparation of the immunoaffinity-purified polyclonal antibody to the 5α-reductase type 2 isozyme was described in detail previously.[3]

Urine collection

A total of 107 urine samples, from 46 females and 61 males, were collected from patients with institutional review board approval and from volunteers with their consent. The female urine samples were collected to serve as negative controls, whereas the male urine samples were collected from men with no history of prostate cancer and from men with a history of prostate cancer. All urine samples were immediately refrigerated upon collection and sent

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to the laboratory coded so that laboratory personnel could not identify the urine with gender or disease state, if any. After laboratory analysis, all urine samples were stored frozen for future reference.

Detection of 5α-reductase type 2 in urine by Western blot

Using the Novex Mini-Cell X Cell II (San Diego, CA) electrophoresis system, 7.5 μl of urine diluted with an equal volume of sample buffer (Novex LC 1676) were applied to each lane of a 10-lane 10–20% tricine precast gel (Novex LC 6625). The CAKP peptide was used as a positive control in one lane. Separation and blotting procedures have been described previously.[3]

Detection of 5α-reductase type 2 in urine by dot blot

Circles with a diameter of 1 cm and with a minimum separation of 1 cm between circles are inscribed with a pencil on a 3-inch square nitrocellulose membrane (Novex LC2001 or equivalent). Apply 7.5 μl of CAKP peptide (100 μg/mL) to one circle as a positive control and 7.5 μl of test urine to each of the remaining circles, keeping each blot within a 5-mm diameter. The membrane is dried gently with a warm stream of air and transferred to a Lab-Tek square Petri dish (96-mm square by 16-mm deep) containing 10 ml of blot buffer (5% carnation non-fat dry milk in phosphate-buffered saline (PBS) and incubated for 1 h at 37°C. The membrane is rinsed with PBS-Tween (0.05% Tween 20 in PBS, pH 7.4) several times and then incubated with 10 ml of antibody (4 μg/mL in blot buffer) for 2 h at room temperature with rocking. After rinsing several times with PBS-Tween, the membrane was incubated sequentially with the same immunoglobulin alkaline phosphatase conjugate and substrate as described for the Western blot.[3] The development of a blue dot indicates a positive finding.

Detection of 5α-reductase type 2 by ELISA

Prepare stock solution of 1 μg/ml CAKP peptide standard in carbonate/bicarbonate buffer, pH 9.6, and working standard solutions of 1, 5, 10, 15, and 20 ng/ml from stock to prepare a standard curve shown in Figure 1. The optimal detection range in this enzyme-linked immunosorbent assay (ELISA) procedure with the antibody used in these studies is between 1 and 20 ng/mL CAKP peptide. Add 100 μL of carbonate/bicarbonate buffer to the blank well and 100 μL of working standards and either test sera or test urines at desired dilutions to the remaining wells of a 96-well Corning disposable sterile ELISA plate. Cover the plate and incubate for 2 h at 37°C and then incubate overnight at 4°C. In one motion, invert the plate and fling the contents of the plate into a waste receptacle and then rap the plate against paper towels several times to remove excess solution. Wash all wells with PBS/0.05% Tween 20 three times, removing solution each time as described previously. Add 200 μL of 1% BSA/PBS to all wells, cover the plate, and incubate for 2 h at 37°C. Wash all wells with PBS/Tween 20 as described previously and add 100 μL of the antibody to the CAKP peptide diluted with PBS/Tween 20 to a concentration of 10 μg/ml. Cover the plate and incubate for 90 min at 37°C. Wash all wells with PBS/Tween 20 as described previously and add 100 μL of anti-rabbit IgG alkaline phosphate conjugate (Sigma A3687) diluted 1:30,000 in PBS/Tween 20. Cover the plate and incubate for 90 min at 37°C. Add 100 μL of p-nitrophenyl phosphate substrate (Sigma Fast pNPP substrate tablet set, N-2770) and incubate for 90 min at 37°C. Read developed color at 405 nm in a microplate reader (Bio-Tek Instruments Kinetic Reader Model EL 312E) and calculate the concentrations from a standard curve.

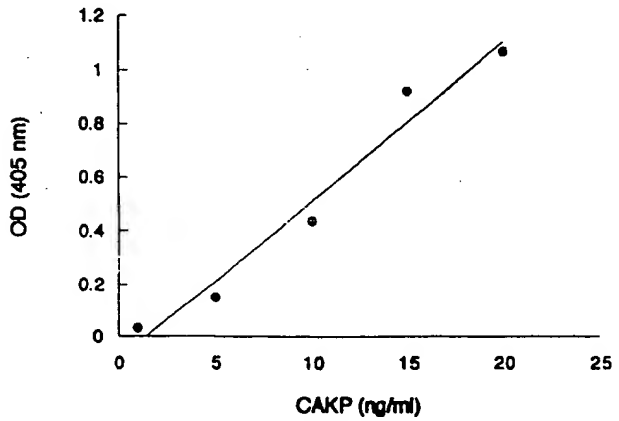

Figure 1 Enzyme-linked immunosorbent assay antibody response curve to CAKP peptide.

Results

Western blot and DOT BLOT analysis of urine for 5α-reductase type 2

The summary of the Western blot analysis is shown in Table 1. There was a total of 46 women in the female control group, 26 women in the premenopausal group and 20 in the postmenopausal group. Forty-five of the 46 women tested negative; the only female testing positive was a 33-year-old woman with nephritis. There was a total of 45 men in the male control group which tested negative. These men had normal prostate-specific antigens (PSAs) and no history of prostate disease. There was a total of 16 men in the male test group. Four men in this group with adenocarcinoma of the prostate, currently being treated with lupron/flutamide to contain the disease, tested negative. Four males with adenocarcinoma of the prostate, not undergoing treatment, tested positive. Two males with transitional cell carcinoma invading the prostatic ducts tested positive, and two males with basal cell hyperplasia of the prostate with intraductal dysplasia also tested positive. Four males with elevated PSAs and no pathology report tested positive. The latter group will be followed clinically and monitored. Testing of all urines by the dot blot procedure confirmed the results of the Western blot in all cases. All positive urine samples were retrieved from frozen storage, centrifuged at 16,000 × *g* to remove cellular debris or free floating protein, and reanalyzed by Western blot. All initially positive assays were confirmed to be positive.

Quantitative analysis of urine for 5α-reductase type 2 by ELISA

The 12 positives in Male Test Group C (see Table 1) were quantitated by ELISA. Fifteen subjects taken at random,

Table 1 Western blot summary of 5α-reductase assay in urine

	No.	Age group	Negative	Positive	Comments
A. Female control group					
	25	29–50	25	0	Premenopausal
	20	51–81	20	0	Postmenopausal
	1	33	0	1	Nephritis
Total	46		45	1	
B. Male control group					
	3	6–12	3	0	None
	38	46–83	38	0	Normal PSA
	4	53–63	4	0	BPH[a]
Total	45		45	0	
C. Male test group					
	4	68–80	4	0	[b]
	4	63–83	0	4	[c]
	2	72,75	0	2	[d]
	2	83,85	0	2	[e]
	4	66–78	0	4	[f]
Total	16		4	12	

[a] BPH, benign prostatic hypertrophy.
[b] Under treatment with lupron/flutamide for adenocarcinoma of the prostate.
[c] Adenocarcinoma of the prostate, elevated PSA, untreated.
[d] Transitional cell carcinoma invading prostatic ducts.
[e] Basal cell hyperplasia with interductal dysplasia.
[f] Significantly elevated PSAs but no pathology report available; patients are being monitored.

eight females from Group A and seven males from Group B (see Table 1), whose urines tested negative by Western blot were used as controls. The CAKP peptide was used as the reference standard for the assay to develop an antibody response curve (Figure 1) to the CAKP peptide. The results of the ELISA assay are shown in Table 2. The ELISA assay confirms the presence of 5α-reductase in the urine of all patients that tested positive by Western blot. The average optical density of the 15 urine samples that tested negative was 0.026 ± 0.003, which represents a concentration of CAKP peptide of less than 0.5 ng/mL.

Discussion

In the last decade, significant progress has been made in the discovery of new tumor markers that have advanced our knowledge in the management of prostate cancer. More significantly, the quantitation of PSA in the peripheral circulation has been one of the most exciting. Currently, however, absolute specificity for any tumor marker for prostate cancer is lacking, and therefore the search must go on. The study reported here was a natural sequence to a previous study reported from our laboratory reporting the detection of the 5α-reductase type 2 isozyme in the serum of patients with prostatic disease.[3]

Urine samples collected from 107 patients, 46 females and 61 males, were examined for the presence of trace levels of the 5α-reductase type 2 isozyme by Western blot,

Table 2 ELISA in urine

Urines positive by Western blot[a]	Optical density at 405 nm	ng/ml
U4	0.110	3.7
M21	0.191	5.8
X1	0.223	6.4
XXX1	0.208	6.1
XXX2	0.166	5.2
XXX5	0.207	6.1
XX7	0.818	14.0
XX9	0.157	5.0
XX10	0.183	5.6
XX11	0.173	5.4
XX12	0.095	3.4
XX14	0.553	11.4
CONTROLS (15)[b]	0.026 ± 0.003[c]	<0.5

[a] Twelve males who tested positive by Western blot taken from Test Group C in Table 1.
[b] Taken at random from urines that tested negative by Western blot, eight females from Group A and seven males from Group B in Table 1.
[c] Mean ± SEM.

dot blot, and ELISA assays. With the exception of a positive Western blot on a urine sample from a patient with nephritis that necessitates further investigation, the data presented here provide strong evidence for the detection of this isozyme in the urine of patients with untreated prostate cancer. The possible use of the Western blot or simple dot blot and ELISA assays for 5α-reductase type 2 in urine as a potential marker for prostate disease would be subject to a well-designed clinical study on a much larger scale than presented here. Although enzyme activity was not used as a criteria in these studies to characterize the 5α-reductase type 2 isozyme, the use of a peptide segment of a protein or enzyme to generate antibodies to that protein or enzyme and the subsequent use of the immunoaffinity-purified antibodies to recognize that protein or enzyme in biological media with a high degree of probability is a well-accepted procedure in the scientific community.[5]

Acknowledgments

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Review

Detection of Prostate Cancer and Predicting Progression: Current and Future Diagnostic Markers

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ABSTRACT

Carcinoma of the prostate is the second leading cause of male cancer-related death in the United States. Better indicators of prostate cancer presence and progression are needed to avoid unnecessary treatment, predict disease course, and develop more effective therapy. Numerous molecular markers have been described in human serum, urine, seminal fluid, and histological specimens that exhibit varying capacities to detect prostate cancer and predict disease course. However, to date, few of these markers have been adequately validated for clinical use. The purpose of this review is to examine the current status of these markers in prostate cancer and to assess the diagnostic potential for future markers from identified genes and molecules that display loss, mutation, or alteration in expression between tumor and normal prostate tissues. In this review we cite 91 molecular markers that display some level of correlation with prostate cancer presence, disease progression, cancer recurrence, prediction of response to therapy, and/or disease-free survival. We suggest criteria to consider when selecting a marker for further development as a clinical tool and discuss five examples of markers (chromogranin A, glutathione *S*-transferase π 1, prostate stem cell antigen, prostate-specific membrane antigen, and telomerase reverse transcriptase) that fulfill some of these criteria. Finally, we discuss how to conduct evaluations of candidate prostate cancer markers and some of the issues involved in the validation process.

INTRODUCTION

Carcinoma of the prostate is the second leading cause of male cancer-related death in the United States, and it is estimated that in 2003 there were approximately 220,900 new cases and 28,900 deaths from this disease (1). Since the introduction of serum prostate-specific antigen (PSA) screening of asymptomatic populations, prostate cancer incidence rates have increased dramatically, as has the number of men undergoing radical prostatectomy and radiation therapy for this disease (1, 2). However, false positives for PSA continue to be a significant problem resulting in unnecessary biopsies, and the value of broad-based PSA testing with regard to predicting surgical cures has recently come into question (3).

Currently, there are no markers that differentiate clinically relevant from clinically benign disease. Better indicators of prostate cancer presence and progression are needed to avoid unnecessary treatment, predict disease course, and develop more effective therapy. A variety of putative prostate cancer markers have been described in human serum, urine, seminal fluid, and histological specimens. These markers exhibit varying capacities to detect prostate cancer and to predict disease course. These markers are distinct from chromosomal aberrations that have been associated with prostate cancer, which will not be dealt with here (4).

The purpose of this review is to examine the current status of markers in prostate cancer and to assess the diagnostic potential for future markers from identified genes and molecules that display loss, mutation, or alteration in expression between tumor and normal prostate tissues. To date, few of these markers have achieved widespread clinical utility. If we are to improve on the treatment of prostate cancer in the 21st century, we must identify and develop markers that are more clinically informative for this disease and that will allow risk-based individualization of therapy.

A BRIEF HISTORY OF PROSTATE CANCER DIAGNOSTICS

The first documented case of prostate cancer was reported by Langstaff in 1817 (5). One hundred eighteen years later, in 1935, prostatic acid phosphatase (PAP) levels were identified in the ejaculate of men, thus linking this enzyme to the prostate (6). Subsequent studies showed high PAP concentrations in primary and metastatic prostate cancer tissues and in human serum, making it the first candidate marker for the diagnosis of prostate cancer (7, 8). Reductions in serum PAP levels were found to occur in response to antiandrogen therapy, whereas increasing serum levels were associated with treatment failure and relapse (9, 10). However, whereas serum PAP levels were elevated in a significant number of men with metastatic disease (8), fewer than 20% of men with localized prostate cancer exhibited abnormal enzyme levels (11, 12). Meticulous sample collection and preparation were required because both platelets and leukocytes are contaminating sources of acid phosphatases (13) and because PAP activity is rapidly lost at room temperature (14). Development of a radioimmune assay for PAP in 1975 provided some improvement in test sensitivity (15), but the sensitivity levels were still inadequate for detection of early-stage disease. Therefore, it was clear that a more sensitive and robust indicator

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of disease presence would be required to detect prostate cancer in its earlier stages, when cure is more likely.

PSA is a kallekrein-like serine protease that was first described in 1971 (16). PSA is secreted from prostate epithelial cells and encoded by an androgen-responsive gene located on chromosome 19q13.3–13.4 (17). The main function of PSA is to liquefy human semen through its proteolytic action (18). PSA was initially thought to be a prostate-specific protein; however, subsequent investigations demonstrated that PSA is secreted in small quantities from a number of other normal male tissues and even some female tissues (19, 20). PSA was first detected in the serum of prostate cancer patients in 1980 (19), and a normal PSA serum concentration limit of 4 ng/ml for men was subsequently established (20). A serum level above 4 ng/ml was taken as an indicator of the possible presence of prostate cancer and served as the trigger for further clinical evaluation. Eventually, a number of studies enrolling large numbers of men over the age of 50 years suggested that quantitation of serum PSA was a useful diagnostic tool for detecting the presence of prostate cancer, particularly when combined with digital rectal examination (21–24). However, other studies have called into question the sensitivity and specificity of the PSA test (25–28). One problem is that serum PSA levels can be elevated as a result of conditions other than prostate cancer, such as benign prostatic hypertrophy (BPH) and prostatitis. As a result, false positives are a significant problem for the PSA test and can lead to unnecessary biopsies and other interventions. Of greater concern, 20–30% of men with prostate cancer have serum PSA levels in the normal range, resulting in undiagnosed disease (22–24). A recent study by Stamey *et al.* (3) has concluded that preoperative serum PSA levels do not correlate with cancer volume or the Gleason grade of radical prostatectomy specimens. This study also showed a poor correlation between preoperative serum PSA levels in the 2–9 ng/ml range and prostate cancer cure rates. Despite the drawbacks and criticisms cited here, PSA is currently the best clinical marker available for prostate cancer and the only one approved by the United States Food and Drug Administration for both posttreatment monitoring of disease recurrence and, when combined with digital rectal examination, evaluation of asymptomatic men (29, 30).

GENES AND PROTEINS CORRELATING WITH PROSTATE CANCER PRESENCE AND PROGRESSION

At the direction of the United States Congress and spearheaded by the National Cancer Institute, support for basic and translational research in prostate cancer has expanded dramatically since 1992. This has resulted in an avalanche of data, much of it attempting to correlate various gene and protein markers with prostate cancer presence, progression, or disease-free survival. Some of these markers have also been proposed as potential therapeutic targets for prostate cancer treatment. However, to date, none of these candidate markers has been adequately validated for clinical use, and no replacement for PSA is visible on the scientific horizon.

Table 1 provides information on 91 genes and their encoded proteins, all of which have a potential role in prostate carcinogenesis and/or progression. All display some level of correlation with one or more of the following factors: presence of prostate cancer, disease progression, cancer recurrence, prediction of response to therapy, or disease-free survival. Information on these markers was accumulated through literature searches using PubMed and from the GeneCards database of human genes, their products, and their involvement in diseases (31). Evidence for the association of a specific marker with human prostate cancer range from a single publication, as in the case of UROC 28, to thousands of publications, as in the case of PSA. In light of the rapid pace of new marker discovery through the use of comprehensive DNA expression analysis and proteomics, there are no doubt candidate markers missing from this list. However, we have made Table 1 as current as possible, and we hope that it will serve as a resource for the prostate cancer research community. In Table 1, we present 89 proteins (the transcripts for DD3 and PCGEM1 do not contain open reading frames) that have been correlated with some aspect of prostate cancer presence or progression in one or more studies. They are listed in alphabetical order and categorized according to their subcellular location: nucleus; cytoplasm; plasma membrane; cytoskeleton; mitochondria; microsomal membrane; endoplasmic reticulum; lysosome; or secreted. The latest information on chromosomal location and molecular weight is included, along with the most common biochemical function of the protein and its major cellular function. In cases in which the biochemical and/or cellular functions of the proteins remain to be determined, the word "unknown" appears in the appropriate column. Alterations for some of these markers, such as p53 and telomerase reverse transcriptase (TERT), can be associated with specific pathways that clearly impact tumor growth and progression; for others, such as DD3 and PC-1, the causal connection is less clear. This is the case for many of the markers presented in Table 1. Whereas an informative marker need not have a specific function in disease progression (PSA is a good example), such a function is useful for understanding the molecular mechanisms of tumor progression and for developing targeted therapeutic interventions.

POTENTIAL FOR DIAGNOSTIC USE

The markers displayed in Table 1 represent a wide array of biochemical and cellular functions. These functions include those of transcription factor, protease, kinase, phosphatase, protease inhibitor, cyclin-dependent kinase inhibitor, cytokine, reverse transcriptase, racemase, reductase, synthase, hydrolase, RNase, molecular chaperone, nuclear matrix, membrane scaffolding, and an assortment of other binding and permeability control proteins. There are also 9 proteins with unknown or poorly defined biochemical functions.

The question is, which of these 91 molecules, if any, are candidates for advancement "from the laboratory bench to the clinic?" This is a broad question that really has a number of parts. First, on what basis do we select from this growing list of candidate diagnostic markers those to pursue in large-scale validation studies designed to prove clinical usefulness? Second, how should these validation studies be conducted and evaluated? Third, what evidence is required to demonstrate that a new marker provides a defined "value added" to the existing methods of prostate cancer detection and for determining the likeli-

hood of disease progression and recurrence and/or response to a given therapy? Fourth, how can we successfully standardize a putative clinical assay to ensure accurate, consistent results across a broad spectrum of research and/or clinical laboratories? This review will focus on the first two questions because addressing them is a prerequisite for moving on to questions 3 and 4.

MARKER SELECTION CRITERIA

What criteria do we consider when selecting one or more of these potential markers for further development as a clinical tool, and will any of the 89 proteins and 2 transcripts presented in Table 1 satisfy these criteria? The most important item regarding the selection of a candidate marker is the quality of scientific and clinical data supporting its potential utility. These include scientific studies relating the functional role of the gene/protein to the biology of the disease and clinical data linking the candidate marker with disease presence, alterations in stage, response to therapy, and/or overall survival. The marker should be measurable by a robust, reproducible, widely available assay that provides useful information that is readily interpretable by the clinician. The ideal candidate for an early detection or disease monitoring marker would be one that is prostate specific; detectable in an easily accessible biological fluid such as human serum, urine, or prostatic fluid; and able to distinguish between normal, BPH, prostatic intraepithelial neoplasia, and cancerous prostate tissues. In addition, the marker should have sufficiently convincing clinical correlation data from several different laboratories before it is brought forward for large-scale evaluation. Whereas it is unreasonable to expect that any single potential diagnostic marker by itself will be able to fulfill all of these criteria, which molecules in Table 1 are the most promising candidates to become clinically useful diagnostic or monitoring markers, and on what basis should we make our selection?

For a marker to be useful for diagnosis and monitoring of disease, it must be demonstrated that the marker correlates with an outcome of interest, such as disease progression, recurrence, or survival. Analyses should be multivariate and should show that the marker(s) predict the outcome of interest independently of the usually available characteristics, such as stage or grade. These assessments should be conducted on a set of cases with adequate outcome data and a sufficient number of events to allow statistical significance to be evaluated. The introduction of tissue microarrays promises to streamline this process considerably.

In the absence of these supportive data, even the most promising marker will not convince either the clinical or pharmaceutical communities that it is worth substantial investment for further evaluation. Based on an analysis of published reports regarding the candidate markers in Table 1, there are five markers that appear to have a significant volume of convincing supportive data, both biological and clinical, associated with them. There are several other candidate markers that have significant supportive data; however, for the purpose of this review, we will discuss these five as examples: chromogranin A (GRN-A); glutathione *S*-transferase π 1 (GSTP1); prostate stem cell antigen (PSCA); prostate-specific membrane antigen (PSMA); and TERT. Their selection in no way diminishes the potential importance of the other markers in Table 1. Each of the proteins listed in the table has different strengths and weaknesses as a clinical prostate cancer marker, and no doubt proteins other than the ones we focus on here will be brought forward for clinical evaluation in the future.

CANDIDATE MARKERS

To choose a marker for diagnosis or prognosis of disease course to bring forward for large-scale clinical evaluation, it should fulfill several criteria. First, there should be a biological or therapeutic rationale for choosing the marker, or at least a consistent association with disease presence, disease characteristics such as stage, or disease aggressiveness. Second, there should be an assessment of the strength of marker association with disease outcome. Third, the marker should be assessed as an independent predictor in a multivariate analysis. The merits and disadvantages of each of the five candidate markers we have selected for scrutiny within the context of the above criteria are discussed below.

GRN-A. GRN-A is a member of the granin family of proteins and acts as a prohormone, which, after proteolytic processing, results in the generation of multiple peptides with biological activity (32). GRN-A is stored in the dense core secretory granules of most endocrine and neuroendocrine cells and is a marker of neuroendocrine differentiation (33). Whereas serum levels of GRN-A do not accurately distinguish BPH from prostate cancer very well, they do correlate with tumor stage and grade. In addition, this marker has the capability to detect neuroendocrine cells and thus has the potential to identify androgen-independent disease. Serum GRN-A levels exhibit a well-documented rise in late-stage disease and demonstrate a wide prevalence range of 32–71%, depending on the study cited (34–43). Studies involving GRN-A have been conducted in human serum using radioimmune assay or ELISA and in tissue using immunohistochemistry (IHC). Elevated serum levels of GRN-A appear to predict poor prognosis in cases of androgen-independent prostate cancer after endocrine therapy and may be an intermediate marker of early progression for this form of the disease and a possible predictor of early death (44, 45). One study used multivariate analysis to demonstrate a significant association between GRN-A positivity and death from prostate cancer (45). Prostate neuroendocrine cells do not contain androgen receptors or produce PSA; thus hormone-refractory disease could be detected earlier in a population of men with apparently normal PSA levels than is currently possible. Whereas GRN-A does not appear to be prognostic of disease recurrence after radical prostatectomy or radiation therapy (46, 47), one report links elevated serum levels to response to estramustine therapy (48). Two significant weakness of GRN-A as a marker are that not all prostate tumors contain neuroendocrine cells and that GRN-A is unable to detect very early stage disease. However, previous studies suggest that GRN-A is able to monitor treatment success, predict disease outcome, and predict prognosis in androgen-independent prostate cancer. There are statistically significant data suggesting that when combined with PSA, elevated GRN-A levels may effectively predict a poor prognosis after endocrine therapy (49). Taken together, this evidence

Table 1 Potential prostate cancer markers

Marker	Chromosome locus	M_r[a]	Subcellular location	Biochemical function	Biological/cellular function
A2M	12p13.3-12.3	163	Secreted	Protease inhibitor	Protein carrier
Akt-1	14q32.32	56	Nucleus/cytoplasm	Protein kinase	Apoptotic inhibition
AMACR	5p13.2-q11	42	Mitochondria/peroxisome	Racemase	Stereoisomerization
Annexin 2	1q21	11	Plasma membrane	Calcium and lipid binding	Membrane trafficking
Bax	19q13.3-.4	21	Cytoplasm/membrane	Bcl-2 binding	Apoptosis
Bcl-2	18q21.3	26	Mitochondrial membrane	Membrane permeability	Apoptosis
Cadherin-1	16q22.1	97	Plasma membrane	Catenin/integrin binding	Cell adhesion
Caspase 8	2q33-34	55	Cytoplasm	Protease	Apoptosis
Catenin	5q31	100	Cytoskeleton	Cadherin binding	Cell adhesion
Cav-1	7q31.1	20	Plasma membrane	Scaffolding	Endocytosis/signaling
CD34	1q32	41	Plasma membrane	Scaffolding	Cell adhesion
CD44	11p13	82	Plasma membrane	Hyaluronate binding	Cell adhesion
Clarl	19q13.3-.4	34	Nucleus	SH3 binding	Unknown
Cox-2	1q25.2-.3	69	Microsomal membrane	Prostaglandin synthase	Inflammatory response
CTSB	8p23.1	38	Lysosome	Protease	Protein turnover
Cyclin D1	11q13	34	Nucleus	CDK[b] regulation	Cell cycle
DD3	9q21-22	0	Nucleus/cytoplasm	Noncoding	Unknown
DRG-1	22q12.2	43	Cytoplasm	GTP binding	Cell growth/differentiation
EGFR	7p12	134	Plasma membrane	EGF binding	Signaling
EphA2	1p36	11	Plasma membrane	Tyrosine kinase	Signaling
ERGL	15q22-23	57	Plasma membrane	Lectin/mannose binding	Unknown
ETK/BMK	Xp22.2	78	Cytoplasm	Tyrosine kinase	Signaling
EZH2	7q36.1	85	Nucleus	Transcription repressor	Homeotic gene regulation
Fas	11q13.3	23	Plasma membrane	Caspase recruitment	Apoptosis
GDEP	4q21.1	4[c]	Unknown	Unknown	Unknown
GRN-A	14q32	50	Secretory granules	Statin	Endocrine function
GRP78	9q33.3	72	Endoplasmic reticulum	Multimeric protein assembly	Cell stress response
GSTP1	11q13	23	Cytoplasm	Glutathione reduction	DNA protection
Hepsin	19q11-13.2	45	Plasma membrane	Serine protease	Cell growth/morphology
Her-2/Neu	17q21.1	138	Plasma membrane	Tyrosine kinase	Signaling
HSP27	7q11.23	23	Cytoplasm	Chaperone	Cell stress response
HSP70	6p21.3	70	Cytoplasm	Chaperone	Cell stress response
HSP90	11q13	63	Cytoplasm	Chaperone	Cell stress response
Id-1	20q11.1	16	Nucleus	Transcription factor	Differentiation regulator
IGF-1	12q22-23	17	Secreted	IGFR ligand	Signaling
IGF-2	11p15.5	20	Secreted	IGFR ligand	Signaling
IGFBP-2	2q33-34	35	Secreted	IGF binding	Signaling
IGFBP-3	7p13-12	32	Secreted	IGF binding	Signaling/apoptosis
IL-6	7p15.3	24	Secreted	Cytokine	B-cell differentiation
IL-8	4q13.3	11	Secreted	Cytokine	Neutrophil activation
KAI1	11p11.2	30	Plasma membrane	CD4/CD8 binding	Signaling
Ki67	10q25-ter	358	Nucleus	Nuclear matrix associated	Cell proliferation
KLF6	10p15	32	Nucleus	Transcription factor	B-cell development
KLK2	19q13.41	29	Secreted	Protease	Met-Lys/Ser-Arg cleavage
Maspin	18q21.3	42	Extracellular	Protease inhibitor	Cell invasion suppressor
MSR1	8p22	50	Plasma membrane	LDL receptor	Endocytosis
MXI1	10q25.2	26	Nucleus	Transcription factor	Myc suppression
MYC	8q24.12-.13	49	Nucleus	Transcription factor	Cell proliferation
NF-kappaB	10q24	97	Nucleus	Transcription factor	Immune response
NKX3.1	8p21	26	Nucleus	Transcription factor	Cell proliferation
OPN	4q22.1	35	Secreted	Integrin binding	Cell-matrix interaction
p16	9p21	17	Nucleus	CDK inhibitor	Cell cycle
p21	6p21.2	18	Nucleus	CDK inhibitor	Cell cycle
p27	12p13.1-12	22	Nucleus	CDK inhibitor	Cell cycle
p53	17p13.1	44	Nucleus	Transcription factor	Growth arrest/apoptosis
PAP	3q21-23	45	Secreted	Tyrosine phosphatase	Signaling
PART-1	5q12.1	7	Nucleus/cytoplasm	Unknown	Unknown
PATE	11q24.2	14	Plasma membrane	Unknown	Unknown
PC-1	5q35	32	Nucleus	RNA binding	Ribosome transport
PCGEM1	2q32	0	Nucleus/cytoplasm	Noncoding	Cell proliferation/survival
PCTA-1	1q42-43	36	Cytoplasm	Unknown	Cell adhesion
PDEF	6p21.31	38	Nucleus	Transcription factor	PSA promoter binding
PI3K p85	5q12-13	84	Cytoplasm	Lipid kinase	Signaling
PI3K p110	1p36.2	120	Cytoplasm	Lipid kinase	Signaling
PIM-1	6p21.2	36	Cytoplasm	Protein kinase	Cell differentiation/survival
PMEPA-1	20q13.31-33	32	Plasma membrane	NEDD4 binding	Growth regulation
PRAC	17q21.3	6	Nucleus	Choline/ethanolamine kinase	Unknown
Prostase	19q13.3-.4	27	Secreted	Serine protease	ECM degradation
Prostasin	16p11.2	36	Plasma membrane	Serine protease	Cell invasion suppressor

Table 1 Continued

Marker	Chromosome locus	M_r[a]	Subcellular location	Biochemical function	Biological/cellular function
Prostein	1q32.1	60[a]	Plasma membrane	Unknown	Unknown
PSA	19q13.3-.4	71	Secreted	Protease	Semen liquification
PSCA	8q24.2	13	Plasma membrane	Unknown	Unknown
PSDR1	14q23-24.3	35[a]	Nucleus/cytoplasm	Dehydrogenase reductase	Steroid metabolism
PSGR	11p15	35	Plasma membrane	Odorant receptor	Unknown
PSMA	11p11.2	84	Plasma membrane	Folate hydrolase	Cell stress response
PSP94	10q11.23	13	Secreted	FSH inhibitor	Growth inhibition
PTEN	10q23.3	47	Cytoplasm	Protein/lipid phopatase	Signaling
RASSF1	3p21.31	33	Cytoplasm	Ras binding	Signaling
RB1	13q14.2	106	Nucleus	E2F-1 inactivation	Cell cycle
RNAseL	1q25.3	84	Cytoplasm/mitochondria	RNAse	Viral resistance
RTVP-1	12q21.1	29	Plasma membrane	Unknown	Immune response/apoptosis
ST7	7q31.2	60/85	Plasma membrane	Unknown	Cell proliferation
STEAP	7q21.23	40	Plasma membrane	Unknown	Unknown
TERT	5p15.33	127	Nucleus	Reverse transcriptase	Telomere synthesis
TIMP 1	Xp11.3-.23	23	Secreted	Protease inhibitor	Cell adhesion
TIMP 2	17q25	24	Secreted	Protease inhibitor	Cell adhesion
TMPRSS2	21q22.3	54	Plasma membrane	Serine protease	Unknown
TRPM2	8p21-12	52	Plasma membrane	Calcium channel	Ion flux
Trp-p8	2q37.1	120	Plasma membrane	Calcium channel	Ion flux
UROC28	6q23.3	17	Nucleus/cytoplasm	Choline/ethanolamine kinase	Unknown
VEGF	6p12	27	Secreted	VEGFR binding	Angiogenesis

[a] Molecular weight (in thousands) estimated from amino acid data.

[b] CDK, cyclin-dependent kinase; EGF, epidermal growth factor; IGFR, insulin-like growth factor receptor; IGF, insulin-like growth factor; LDL, low-density lipoprotein; ECM, extracellular matrix; FSH, follicle-stimulating hormone; VEGFR, vascular endothelial growth factor receptor.

[c] Data for Table 1 resourced from GeneCards database, Weizmann Institute of Science (31).

makes GRN-A a good candidate for further clinical evaluation as a prognostic and/or treatment marker for prostate cancer.

GSTP1. GSTP1 is a member of a large family of glutathione transferases that function to protect cells from oxidative insult (50); thus, the biological rationale for selecting this marker is its role in preventing damage to cells by neutralizing free radicals. This marker is also unique in its capacity to provide a facile methylation-based detection method for an important epigenetic phenomenon. GSTP1 has been extensively studied in prostate cancer, and its reduced expression, due predominantly to promotor hypermethylation, represents the most common epigenetic alteration associated with this disease. One study has shown that in prostate cancer cells, methylation of the *GSTP1* gene is not confined to the promoter but is extensive throughout the CpG islands (51). Several studies have shown a high sensitivity for this marker to detect the presence of both prostatic intraepithelial neoplasia and prostate cancer, an ability to distinguish these from BPH, and a prevalence of methylation in the range of 60–80% in prostate cancer (51–61). In addition, several *GSTP1* polymorphisms have shown a correlation with increased risk of disease development, although data regarding this ability are conflicting (62–67). Strengths of GSTP1 as a clinical marker are the ability to quantitate the methylation status of the *GSTP1* gene in biopsy/prostatectomy tissues and in cells derived from serum, urine, and seminal plasma and its high prevalence in this disease. Recent studies using quantitative real-time methylation-sensitive PCR demonstrate that *GSTP1* methylation could be a sensitive marker for prostate cancer in men with clinically localized disease (51). In addition, there is no correlation between *GSTP1* methylation status and PSA levels, making GSTP1 a potential early and independent marker for the disease. The ability of *GSTP1* hypermethylation to distinguish between BPH and prostate cancer is well documented, and one recent study correlated methylation status with poor prognosis in 101 patients diagnosed with prostate cancer (68). However, whereas these results are statistically significant, they were not tested by multivariate analysis. Reversal of *GSTP1* CpG island hypermethylation and gene reactivation in LNCaP prostate carcinoma cells can be achieved by procainamide treatment; however, no effect on tumor cell growth was observed in these studies (69). The strengths of *GSTP1* methylation status, as cited above, and the possible availability in the near future of drugs that can reverse hypermethylation make it a good candidate for further evaluation as an early detection marker. If successfully validated, *GSTP1* methylation testing of cells derived from serum and urine samples may have clinical usefulness for both early detection of prostate cancer and posttreatment monitoring of disease.

PSCA. PSCA is a glycosylphosphatidylinositol-anchored cell surface antigen that is found predominantly in prostate and may play a role in stem cell functions such as proliferation or signal transduction (70). Whereas the biological role of PSCA in prostate cancer is unclear, the marker is expressed predominantly in the prostate and has potential as a therapeutic target. Other strengths of PSCA as a prostate cancer marker include elevated PSCA expression levels in the majority of prostate cancers and a correlation between this elevation and higher Gleason grade and more advanced tumor stage (71–74). Published studies also show a high correlation (64–94%) between increased PSCA expression and the presence of prostate cancer, with protein expression localized to both the basal and secretory cells (71–75). PSCA has been assayed by a variety of methods, including *in situ* hybridization, quantitative reverse transcription-PCR, and IHC, demonstrating a prevalence of 48–94% for

prostate cancer (71, 72, 76). One IHC study demonstrated an association between increased PSCA expression and higher Gleason score, more advanced tumor stage, and progression to androgen-independent prostate cancer (72). However, extensive multivariate analysis to confirm these findings has yet to be performed. PSCA expression is maintained in androgen-independent prostate cancer, and PSCA is highly expressed in metastatic disease (71–76). Whereas most of the studies performed to date have been on prostate tissue samples, there is at least one report of PSCA detection in peripheral blood (73). Another strength of this marker is its potential as a therapeutic target. Anti-PSCA monoclonal antibodies have been shown to inhibit tumor growth and metastasis formation of human xenografts grown in *scid* mice (76). This opens up the possibility for therapeutic treatment of human prostate cancers using immunotherapeutic regimens (76–78). In addition, PSCA is co-amplified with the tumor progression factor and oncogene c-*myc* in locally advanced prostate cancers, suggesting a role for PSCA in the progression of this disease (74, 79). Three weaknesses of PSCA as a candidate for further development are the limited number of published studies supporting its value as a clinical marker, a need for better quantitation methods, and uncertainty as to whether analysis of PSCA levels adds information to the results of PSA testing. However, based on the available data and the value of PSCA as a therapeutic target, further evaluation of PSCA as a clinical prostate cancer marker should be performed to determine its utility.

PSMA. Discovered in 1987, PSMA is a cell surface membrane protein and one of the most extensively studied prostate cancer markers cited in Table 1 (80). PSMA is a type II integral membrane protein that displays multiple enzymatic activities (81, 82). The protein translocates from the cytosol in normal prostate to the plasma membrane in prostate cancer (83). The exact biological role of PSMA in the disease mechanism is unclear at this time; however, extensive data exist on its utility as a marker and therapeutic target. Numerous studies have shown that PSMA serum levels are elevated in primary prostate cancer and metastatic disease, that PSMA demonstrates a >90% prevalence in the disease, and that levels can be detected in both tumor tissue and serum using several antibodies (84–91). PSMA has been detected in prostate tissues using IHC and Western analysis, in circulating prostate cancer cells by reverse transcription-PCR, and in serum using ELISA assays. One study using Western analysis demonstrated that in postprostatectomy patients, PSMA values are elevated in hormone-refractory tumors, suggesting that PSMA levels may correspond with poor clinical outcome (85). In another study (92), PSMA serum levels were found to increase with age and were significantly elevated in men over 50 years of age. To date, however, increased PSMA serum levels have not been convincingly linked to disease aggressiveness, and perhaps due to tumor differentiation status, some studies have shown that levels actually decrease in advanced disease (93). PSMA protein has also been shown to be up-regulated in prostate cancer patients after androgen deprivation therapy (94). Recent technological advances have allowed for the high-throughput assay of this marker in human serum using a protein chip, mass spectrometry platform (95). That study demonstrated significantly greater PSMA levels in men with prostate cancer than in those with BPH or with no evidence of disease. PSMA is moderately prostate specific and has been investigated as a target for immunotherapy using autologous dendritic cells (96). Efforts are also under way using the PSMA gene promoter to pursue gene therapy strategies by introducing cytotoxic agents into prostate cancer cells (97). A weakness of PSMA as a clinical marker for early diagnosis is that elevated serum levels have been observed in healthy males and females and in the serum of breast cancer patients (98). Another weakness is that serum levels of PSMA have been shown to increase with increasing age, which could be a confounding factor in a disease that most often occurs late in life. However, there is an abundance of data supporting the ability of PSMA to detect the presence of prostate cancer, and new technologies are being developed that allow quantitative high-throughput analysis of biological fluids. This argues in favor of further evaluation of this marker to determine whether or not it has clinical utility for prostate cancer detection or treatment monitoring or as a treatment target.

TERT. The *TERT* gene encodes the reverse transcriptase component of telomerase that maintains the telomeric ends of chromosomes and has been associated with senescence and cancer (99). The TERT component is expressed in cells that exhibit telomerase activity and is undetectable in most benign tissues (100). The biological rationale for selecting TERT is the ability of TERT to confer cellular immortalization, a major step in the process of malignant transformation. Thus, this marker may provide a very sensitive means for detecting infiltrating cancer cells in benign tissue. A significant number of studies have been conducted to evaluate TERT or telomerase activity as a marker for prostate cancer (101–119). Published reports demonstrate that TERT activity levels exhibit a prevalence range of 63–94% for prostate cancer, and activity has been detected in some cases of high-grade prostatic intraepithelial neoplasia (100–111). TERT has been most often assayed by IHC or the telomeric repeat amplification protocol assay. Most studies find the marker consistently absent from normal prostate and the majority of BPH tissues. The highest TERT activity appears to correlate with poorly differentiated disease, and there is some evidence for a correlation with tumor stage and grade and patient mortality and disease recurrence (100, 104, 107–109). Whereas statistical significance has been demonstrated in some of these studies, the correlations have not been tested by multivariate analysis. TERT activity does not correlate with PSA level, making it a potential independent marker for prostate cancer. One study suggests that telomere length in tumor tissues correlates with survival and recurrence in prostate cancer patients (120). However, TERT also displays several weaknesses as a clinical marker for prostate cancer detection. TERT is not prostate specific, and in most studies, assays were conducted in prostate tissues, and thus required biopsy material before marker assay. However, several studies have now successfully used human urine, seminal fluid, and prostatic fluid to detect TERT activity (113, 114, 121). Although TERT activity levels appear to be independent of PSA, the "value added" of TERT for early detection, staging, or prognosis and the overall clinical utility of TERT remain to be fully uncovered. Further evaluation of TERT may reveal a niche for use of TERT as a supplement to PSA testing.

MARKERS IDENTIFIED BY ADVANCED TECHNOLOGIES

Recently, methodologies as diverse as positional cloning, differential display, comprehensive DNA expression analysis, and serum proteomic analysis have provided some very preliminary yet exciting prostate cancer marker candidates. Among these are Hepsin, a serine protease associated with cell growth and morphology (122–124); RNase L, a RNase involved in viral resistance and a candidate for the HPC1 gene (125, 126); ST7, a protein of unknown function (127–129); and EZH2, a homeotic protein that participates in the repression of gene expression (130, 131). In addition, proteomic analysis of serum from prostate cancer patients has shown promise for diagnostic and prognostic use for this disease (132). Candidate markers identified by these new technologies will require confirmation and correlation with disease formation or progression or with patient survival or response to therapy in additional human samples before they can be considered for validation studies.

CLINICAL EVALUATION AND AN EVALUATION PROCESS EXAMPLE

To validate the clinical usefulness of any marker, it is important to first establish what the end point will be. This, in turn, will determine the study population that will be examined. The appropriate statistical design of the study will require information on prevalence and the postulated strength of the association of marker expression with the outcome of interest. These considerations will, in turn, determine both specificity and sensitivity of the marker. In some situations, an appropriate control population may be required to determine the specificity of the marker through the determination of false positive and negative rates. Finally, an appropriate sample collection, preparation, and assay method must be decided on. Multi-institutional clinical studies will also likely require the use of a central laboratory or shared controls and training sets of malignant samples to ensure accuracy and consistency. An example may serve to illustrate a reasonable evaluation process.

Suppose we wish to further evaluate a hypothetical marker, which we will call Optimal Marker in Prostate Carcinoma (OMPC), for clinical utility. Previous studies indicate a correlation between OMPC expression and metastatic disease. The clinical question to be addressed is whether this marker can predict which patients with early-stage disease will eventually develop metastatic disease despite local therapy. To accomplish this, we would want to evaluate a large enough number of cases of early-stage prostate cancer for which there is a minimum of 5 years of clinical follow-up data available to obtain a robust assessment of the strength of association between the marker and outcome. The method of OMPC analysis would be selected based on limit of detection, sources of variability, suitability for the samples that will be available, day-to-day and interobserver variability, cost, scalability, and optimum reagents (133). The procedure for scoring or interpreting the results of the assay will need to be optimized to reduce variability. If cut points are to be used, they should also be developed and tested for robustness, if necessary.

The optimized assay is then performed on a large number of early-stage prostate cancer cases, with the hypothetical result that all patients express the marker, but only 40% express at "high" levels as determined by our cut point. For illustrative purposes, say these high expressers have a 2.5× greater risk of developing metastatic disease after local therapy. Does OMPC predict outcome at least as well as or independently of the other known prognostic factors, Gleason score and PSA? Because all of the patients had early-stage cancers, a multivariate analysis including all of the significant prognostic factors would answer this question. If OMPC remained a strong prognostic factor for development of metastatic disease, with a hazard ratio of at least 2, and was independent of other prognostic factors, we would be more confident of its potential value. However, this would require verification in an independent cohort of patients with early-stage prostate cancer receiving a defined initial local treatment. Using an estimate of 40% high expression prevalence, we can calculate the size of the study necessary to determine whether the marker is associated with a hazard ratio of at least 2, with an 80% power to detect a minimum 2-fold effect on the likelihood of metastatic development (134). Whereas we have here addressed the analysis of a single marker, the strategy outlined would also apply to a genomic or proteomic "profile" as determined by comprehensive molecular analysis.

CONCLUSIONS

The development of novel and clinically relevant markers for prostate cancer diagnosis, prognosis, and prediction is essential to the optimal identification and treatment of this disease. With the advent of DNA expression analysis, tissue microarrays, and proteomic analysis, the list of potential prostate cancer markers grows daily. Sorting through these potential markers and bringing them from the laboratory environment into clinical use at the patient bedside will require a comprehensive pursuit and rigorous analysis. Many of the molecules cited in Table 1 have languished for years in a gray zone between usefulness as a clinical marker for prostate cancer and elimination from further consideration. As a research community, we must devise approaches that will ensure that we realize the next generation of clinically relevant prostate cancer markers.

We have here attempted to provide examples of potential prostate cancer markers that may be of clinical benefit in prostate cancer detection, prognosis, and/or prediction. We have also suggested one possible methodology for the clinical evaluation of these markers. The goal of this effort is not to dictate the optimal markers or the methodologies for their verification, but to provide by example a framework from which the general research community can work toward achieving the goal of bringing new prostate cancer markers forward for clinical use.

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